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Case Report

Stability of plasma adrenocorticotrophic hormone (ACTH): Influence of hemolysis, rapid chilling, time, and the addition of a maleimide

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ABSTRACT

Objectives: The aim of this study was to examine the effects of hemolysis, rapid chilling, time, and the addition of a maleimide on the stability of human plasma ACTH measurements.

Design and methods: Partially hemolyzed EDTA blood ($n=10$), initially at 37 °C, was centrifuged at 4 °C either immediately or after rapid chilling in ice/water. Plasma ACTH was then measured either immediately, or after 1 h at 22 °C with or without the addition of 2 mM *N*-phenyl maleimide (NPM).

Results: For 0.2% hemolysis compared to no hemolysis, the mean (±SEM) loss with immediate centrifugation and immediate ACTH measurement was 11 ± 1%. This loss was significantly ($p<0.002$) reduced to 6 ± 1% by an initial rapid chilling of the samples. For analysis after 1 h at 22 °C, the addition of NPM decreased the loss of ACTH from 15 ± 2% to 2 ± 2% ($p<0.002$).

Conclusion: Rapid chilling, prompt analysis, and addition of NPM can each reduce the interference of hemolysis in the measurement of plasma ACTH concentrations.

Introduction

A common pre-analytical problem is hemolysis [1]. This is well documented as causing severe interference with insulin measurements, probably because of proteolysis [2–4], but the effect on other polypeptide hormones is less well quantified.

The measurement of adrenocorticotropic hormone (ACTH) is stated to be sensitive to hemolysis [5], but the only published documentation of this is a mention in an abstract [6]. It has also been recommended that samples for ACTH measurement be chilled to 0 °C immediately after venepuncture [5], and that *N*-ethyl maleimide (NEM) be used to prevent the loss of ACTH in non-hemolyzed plasma [6,7]. We quantify for the first time the loss of ACTH immunoreactivity caused by hemolysis and report on the influence of rapid chilling, standing at room temperature and the addition of a maleimide to the plasma. For comparison, we also measured insulin losses.

Methods

Blood collection and experimental design

Blood was collected from ten healthy volunteers (6 F, 4 M) into 9 mL evacuated tubes containing K_3EDTA (tri-potassium ethylenediamine tetraacetic acid) (Vacuette, Greiner Bio-One, Kremsmunsten, Austria) by specialist nurses using a winged infusion set with a 21-gauge needle, and carried (about 10 min) to the laboratory on ice. For each volunteer, the bulk of the whole blood was pooled and kept on ice while hemolyzed blood was prepared by freezing two 200 µL aliquots of the whole blood at −28 °C for 30 min.

After warming 2 and 4 mL aliquots of the bulk whole blood at 37 °C for 10 min, the hemolyzed blood was added to it to give 0, 0.1%, 0.25%, 0.5%, and 1.0% by volume. Four treatments were then carried out at each level of hemolysis. Aliquots were either (treatment A) immediately centrifuged at 1200 ×*g* for 10 min at 4 °C and 0.5 mL of plasma stored frozen for later analysis or (treatments B, C and D) incubated in an ice/water mixture for 30 min before centrifugation as for treatment A. For treatment D, 2 mM *N*-phenyl maleimide (Sigma-Aldrich, Castle Hill, Australia), as a 400 mM stock solution in dimethyl sulfoxide, was added to the plasma before freezing. One to four days later, after thawing for 6 min in ambient temperature water, the plasma samples were mixed and re-centrifuged as above. Plasmas were then analyzed for ACTH and insulin either immediately

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Table 1
Experimental design: summary of differences between sample treatments.

Treatment	Ice/water post-venepuncture	One hour 22 °C before analysis	NPM (2 mM)
A	−	−	−
B	+	−	−
C	+	+	−
D	+	+	+

(treatments A or B), or after standing for 60 min at room temperature, 21–22 °C (treatments C and D). The designed differences between the four treatments are summarized in Table 1.

Volunteers gave written informed consent, and the study was approved by the Upper South A Regional Ethics Committee.

Analytical methods

Hemoglobin (Hb) was measured using a Sysmex XE-2100 analyzer (Sysmex, Kobe, Japan). ACTH [8] and insulin were measured using the Elecsys 2010 analyzer (Roche, Mannheim, Germany). The respective intra-assay coefficients of variation were 2.9% and 1.9% (manufacturer's data).

Statistical analysis

For each subject and analyte, concentrations (Y) were expressed as a percentage of the non-hemolyzed sample value for treatment B at time zero. Repeated-measures ANOVA (analysis of variance) was performed using NCSS statistical software (NCSS, 329 North 1000 East, Kaysville, Utah 84037, USA), with percentage hemolysis as the within factor and treatment group as the between factor. The Tukey–Kramer and the Kruskal–Wallis multiple-comparison tests [9] were used to compare group means and medians, respectively.

To quantify the effect of hemolysis, for each analyte, subject and treatment the exponential function:

$$Y = ae^{-bH} \quad (1)$$

was fitted where H is the percentage of hemolyzed blood added and a and b are adjustable parameters fitted by least squares using NCSS. The maximum acceptable level of hemolysis was taken as a mean loss of 10% (recovery of 90%), calculated from Eq. (1). The hemoglobin (Hb) concentration in hemolyzed plasma was calculated from the hemoglobin concentration measured in the whole blood.

Results

The mean ± SEM (standard error of the mean) whole blood hemoglobin concentration for the ten volunteers was 142 ± 5 g/L. Their mean plasma ACTH and insulin concentrations in the absence of hemolysis were 4.8 (range 0.8–15) and 75.6 (range 44.1–163) pmol/L, respectively.

Recoveries of ACTH immunoreactivity for differing percentages of hemolysis and the different treatments are plotted in Fig. 1. Repeated-measures ANOVA showed that hemolysis significantly ($p<0.0001$) lowered mean measured ACTH and insulin concentrations and that greater hemolysis resulted in greater losses ($p<0.0001$ for linear trend). The ANOVA also demonstrated that there were significant differences between the four treatment groups for both analytes (both $p<0.0001$).

Multiple-comparison tests found that for both ACTH and insulin, samples chilled in ice/water before centrifugation (treatment B) lost significantly ($p<0.001$) less immunoreactivity than did the samples centrifuged immediately (treatment A). Also, immediate analysis (treatment B) resulted in a significantly ($p<0.001$) lower loss of both

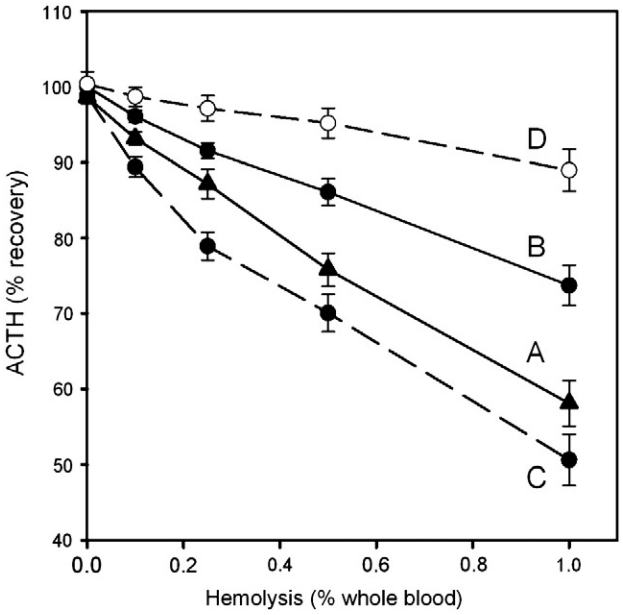

Fig. 1. Effect of level of hemolysis, time, temperature, and addition of 2 mM *N*-phenyl maleimide on measured ACTH concentrations. EDTA whole blood ($n = 10$), to which 0, 0.1%, 0.25%, 0.5%, or 1.0% of hemolyzed whole blood had been added at 37°C, was either chilled in ice/water (circles) or not (triangles), before centrifugation at 4°C. The plasma was then stored frozen either with the addition of 2 mM *N*-phenyl maleimide (open circles), or without (filled symbols). Subsequently, the thawed plasma was either analyzed immediately (solid lines) or after 1 h at 22 °C (dashed lines). Recoveries (mean ± SEM) for the differing sample treatments are shown as ▲——▲ treatment A (−chill, −RT, −NPM), ●——● treatment B (+chill, −RT, −NPM), ●– – –● treatment C (+chill, +RT, −NPM), o– – –o treatment D (+chill, +RT, +NPM), where chill = blood sample chilled at 0°C for 30 min immediately after venepuncture, RT = plasma stood at 22 °C for 1 h before analysis, NPM = 2 mM NPM added to plasma.

ACTH and insulin than occurred when the samples were stood at 22 °C for 1 h before analysis (treatment C). Addition of 2 mM NPM to the plasma (treatment D) significantly ($p<0.001$) decreased the loss of ACTH during 1 h of standing at room temperature when compared to standing without NPM (treatment C).

The recoveries for insulin were similar to those shown in Fig. 1 for ACTH, but mean recoveries were higher for treatments A, B, and C and lower for treatment D ($p<0.001$, <0.005, <0.001, and <0.04, respectively, by multiple-comparison testing.

Levels of hemolysis predicted by Eq. (1) to cause a 10% mean change in apparent concentrations are given in Table 2 for each treatment.

Discussion

This is the first report to quantify the effect of hemolysis on ACTH measurement. Although hemolysis is well known to interfere with ACTH measurement (for example, the Roche kit method sheet for the ACTH method and reference [5]) and this interference was first noted in 1976 [7], there has been no previous report of the magnitude of the effect, nor has the influence of temperature, time, or protease inhibitors been examined.

As the limit of visually reliably detectable hemolysis is about 0.3 g/L Hb [1], it is of concern that we have found that as little as 0.1% hemolysis (0.1–0.2 g/L Hb) can cause significant loss of ACTH (Table 2, treatment C) if the precautions of rapid cooling and prompt analysis are not taken.

We found that rapid cooling of blood at 37 °C in ice/water before centrifugation at 4 °C (treatment B), rather than placing the blood directly in the centrifuge (treatment A), resulted in a significantly reduced loss of ACTH (Fig. 1) and lesser interference from hemolysis

Table 2
Median (95% confidence limits) levels of hemolysis predicted from Eq. (1) to give 90% recovery for ACTH or insulin in EDTA plasma samples. Levels of hemolysis are calculated both as %whole blood hemolyzed and as hemoglobin concentration in plasma. For clarity, statistical significance of differences is given only for the latter. See Table 1 for a summary of the experimental design and the designed differences between treatments A, B, C, and D.

	Treatment A (−chill, −RT, −NPM)		Treatment B (+chill, −RT, −NPM)		Treatment C (+chill, +RT, −NPM)		Treatment D (+chill, +RT, +NPM)	
Units	%	Hb g/L	%	Hb g/L	%	Hb g/L	%	Hb g/L
ACTH	0.20 (0.11–0.25)	0.26[c] (0.15–0.34)	0.31 (0.23–0.65)	0.40[cd] (0.35–0.98)	0.10 (0.04–0.14)	0.14[de] (0.08–0.21)	0.80 (0.47–1.7)	1.13[e] (0.66–2.3)
Insulin	0.27 (0.2–0.36)	0.42[d] (0.26–0.45)	0.61 (0.37–1.07)	0.91[de] (0.56–1.42)	0.20 (0.16–0.37)	0.28[ef] (0.23–0.49)	0.68 (0.46–1.8)	0.97[f] (0.69–2.3)

Significance of planned paired comparisons of medians (row-wise): [c]$p<0.04$; [d]$p<0.005$; [e,f]$p<0.001$. Chill = blood sample chilled at 0 °C for 30 min immediately following venepuncture, RT = plasma stood at 22 °C for 1 h before analysis, NPM = 2 mM NPM added to plasma.

(Table 2). This observation provides support for the hitherto undocumented recommendation in the Roche ACTH method sheet to place freshly taken blood samples immediately on ice.

The kit manufacturer, Roche, also recommend in the ACTH method sheet that samples be at ambient temperature (20–25 °C) before analysis. Our finding that the interference from hemolysis is increased by leaving samples for 1 h at ambient temperature before analysis (compare treatments B and C in Table 2) demonstrates that samples for ACTH measurement must be analyzed immediately on attainment of ambient temperature if interference from even very low levels of hemolysis is to be minimized.

These recommendations contrast with those arising from our earlier work [10] and from that of Reisch and co-workers [11], where ACTH appeared stable for 18–24 h at room temperature in EDTA plasma, and cooling did little to improve stability. However, these studies excluded even slightly hemolyzed samples, which is unrealistic in real laboratory life where such samples are fairly common [1].

Our finding that the addition of NPM to hemolyzed plasma reduces the loss of ACTH at ambient temperature (Fig. 1, treatment D,) is novel and may be of value for stabilizing ACTH in plasma where slight hemolysis is a possibility. Indeed, the reported stabilization of ACTH in plasma by another maleimide, NEM [6,7], might thus be due to the counteracting of visually imperceptible micro-hemolysis.

Maleimides specifically alkylate sulfhydryl groups in the pH range 6.5–7.5 to form stable thioether bonds, while at higher pH values, there is some reactivity with amino groups [12]. They are among the reagents commonly recommended for modification of the cysteine residues of proteins [13] and for the inhibition of cysteine proteases [14], raising the possibility that the cause of the interference of hemolysis in the measurement of ACTH is a cysteine protease released from blood cells.

Unfortunately, we were unable to introduce NPM into whole blood without causing hemolysis, and other common protease inhibitors that may have been more erythrocyte-compatible appeared ineffective for reducing the interference of hemolysis on ACTH measurement (data not shown). Another maleimide, NEM, is also reported to hemolyze whole blood [6]. The inconvenience of adding NPM to plasma probably rules out adding it to routine clinical samples, but it should be considered for research studies where large numbers of ACTH measurements are required to be made under optimal conditions.

Interestingly, we have found ACTH to be even more sensitive to hemolysis than insulin (Table 2), at least in the absence of maleimide.

In conclusion, ACTH measurements can be subject to interference from very low, barely visible, levels of hemolysis (0.1–0.2 g/L Hb). Rapid chilling, prompt analysis, and the addition of a maleimide can minimize this interference. Consequently we recommend that:

1. Blood samples for the measurement of ACTH are rapidly chilled by placement in an ice/water slurry for 15–30 min immediately following venepuncture and before centrifugation at 0–4 °C.
2. Plasma for the measurement of ACTH should not remain at room temperature for more than 15 min before placement on an analyzer.
3. Samples for optimal ACTH measurement in research studies should have 2 mM *N*-phenyl maleimide added immediately after the separation of the plasma from the cells.

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References

[1] Lippi G, Blanckaert N, Bonini P, Green S, Kitchen S, Palicka V, et al. Haemolysis: an overview of the leading cause of unsuitable specimens in clinical laboratories. Clin Chem Lab Med 2008;46:764–72.
[2] Brodal BP. The influence of haemolysis on the radioimmunoassay of insulin. Scand J Clin Lab Investig 1971;28:287–90.
[3] Chevenne D, Letailleur A, Trivin F, Porquet D. Effect of hemolysis on the concentration of insulin in serum determined by RIA and IRMA. Clin Chem 1998; 44:354–6.
[4] Sapin R, Ongagna J-C, Gasser F, Grucker D. Insulin measurements in hemolysed serum: influence of insulinase inhibitors. Clin Chim Acta 1998;274:111–7.
[5] Wilde C. Subject preparation, sample collection and handling, third ed. In: Wild D, editor. The Immunoassay Handbook. Oxford: Elsevier; 2005. p. 443–55.
[6] Hogan P, Rees LH, Lowry PJ, Ratter S, Snitcher EJ. Studies on the stability of human adrenocorticotrophin in blood and plasma. J Endocrinol 1976;71:63p–4p.
[7] Jubiz W, Nolan G. *N*-ethylmaleimide prevents destruction of corticotrophin (ACTH) in plasma. Clin Chem 1978;24:826–7.
[8] Verschraegen I, Anckaert E, Schiettecatte J, Mees M, Garrido A, Hermsen D, et al. Multicentre evaluation of a rapid electrochemiluminescent adrenocortocotropic hormone (ACTH) immunoassay. Clin Chim Acta 2007;380:75–80.
[9] Wheater CP, Cook PA. Using Statistics to Understand the Environment. Abingdon: Routledge; 2000. Chapter 7.
[10] Ellis MJ, Livesey JH, Evans MJ. Hormone stability in whole blood. Clin Biochem 2003;36:109–12.
[11] Reisch N, Reincke M, Bidlingmaier M. Preanalytical stability of adrenocorticotopic hormone depends on time to centrifugation rather than temperature. Clin Chem 2007;53:359–60.
[12] Hermanson GT. Bioconjugate Techniques. 2nd ed. New York: Academic Press; 2008. p. 183–4.
[13] Purich DL. Enzyme Kinetics: Catalysis and Control. New York: Elsevier; 2010. 541 pp.
[14] Sigma-Aldrich. Broad spectrum inhibitors. http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/protease-inhibitors/broad-spectrum-inhib.html (accessed 14 September 2010).